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Human and rat TR4 orphan receptors specify a subclass of the steroid receptor superfamily

(TR2 orphan receptor/prostate/hippocampus/cerebellum)

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ABSTRACT **We have identified a member of the steroid receptor superfamily and cloned it from human and rat hypothalamus, prostate, and testis cDNA libraries. The open reading frame between first ATG and terminator TGA can encode 615 (human) and 596 (rat) amino acids with calculated molecular mass of 67.3 (human) and 65.4 (rat) kDa. The amino acid sequence of this protein, called TR4 orphan receptor, is closely related to the previously identified TR2 orphan receptor. The high homology between TR2 and TR4 orphan receptors suggests that these two orphan receptors constitute a unique subfamily within the steroid receptor superfamily. These two orphan receptors are differentially expressed in rat tissues. Unlike TR2 orphan receptors, the TR4 orphan receptor appears to be predominantly located in granule cells of the hippocampus and the cerebellum, suggesting that it may play some role(s) in transcriptional regulation in these neurons.**

Orphan receptors in the superfamily of steroid hormone receptors share with genuine receptors a common domain architecture and a high degree of identity in the DNA-binding domain (DBD) but have no known ligands (1, 2). Their functions have been elusive and subject to speculation, but recently several potential functions have been explored (2, 3). Ligands for several orphan receptors have been identified and alternative activation mechanisms and functions have been proposed. Retinoid X receptors (RXRs) and peroxisome proliferator-activated receptors (PPARs) can be activated by the retinoid metabolite 9-*cis*-retinoic acid and peroxisome proliferators, respectively (4, 5). Other orphan receptors may have no ligands but can be activated by signal transduction pathways, as has been proposed for TR2 orphan receptor (6, 7). Some are constitutive transactivators, such as TR3/NGFI-B/Nur77 (8, 9), or repressors, such as COUP-TF I and ARP-1 (10–12). An intriguing property of some orphan receptors is their ability to facilitate or modify ligand-mediated signaling by interaction with other members of the steroid hormone receptor superfamily at the protein or DNA level. RXRs are essential for signaling by retinoids, thyroid hormone, vitamin D, and peroxisome proliferators through acting as heterodimerization partners for the respective receptors (13). COUP-TF I also has this potential (11). However, the main route of interference is through direct binding to response elements of receptors for retinoids, thyroid hormone, vitamin D, and peroxisome proliferators (10, 14) and the estrogen receptor when its binding site overlaps with an estrogen response element (15).

These properties make orphan receptors play very important roles in signal transduction in complex cell systems and organs. The nervous system relies on complex cell–cell interactions and may reveal functions of orphan receptors. Indeed, several orphan receptors have been detected in the brain. The orphan receptors TR3/NGFI-B/Nur77 and NurrI were shown to act as immediate early gene products in nervous tissues and cells (8, 9, 16), while COUP-TF was indicated to play a role in the regulation of the oxytocin gene in the hypothalamoneurohypophyseal system and the corpus luteum (15, 17). Since the hypothalamoneurohypophyseal system is a neuronal system of well-known hormonal and neural integration, we set out to identify members of the steroid hormone receptor superfamily in this brain region. Using the hypothalamic supraoptic nucleus (SON) as source for degenerate PCR cloning, we here describe the identification and distribution of a member we have named TR4 orphan receptor.¶ The structure of this receptor reveals that it specifies a distinct subfamily of the steroid hormone receptor superfamily together with the TR2 orphan receptor.

MATERIALS AND METHODS

PCR Cloning of Full-Length Human and Rat TR4 Orphan Receptor cDNAs. Wistar rats (250–300 g) were decapitated and hypothalamic tissue including the SON was microdissected from 2-mm fresh tissue slices with needles of diameter 1 mm (18). Total RNA (2.5 μg) was annealed to 1 μg of random hexanucleotide primers and reverse transcribed with 400 units of SuperScript reverse transcriptase (BRL) in 20 μl of 50 mM Tris·HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$/10 mM dithiothreitol/1.25 mM dNTPs at 37°C for 1.5 hr. Then SuperScript was heat inactivated at 90°C for 5 min, and 30 units of RNase H (Amersham) was added and incubated at 37°C for 20 min. One-tenth of the cDNA was supplemented with 50 pmol of the appropriate 5′ primer and 3′ primer and amplified with 1.0 unit of Replitherm polymerase (Epicentre, Madison) in 50 μl of 50 mM KCl/10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$/0.1% gelatin/200 μM dNTPs. The degenerate primers were directed to the DBD of all steroid hormone receptors except those which contain a P-box with the amino acid sequence Gly-Ser-Cys-Lys-Val (glucocorticoid, mineralocorticoid, progesterone, and androgen receptors). The primers were DBD66-100 (5′ primer), 5′-GGAGTCGGTACCTG(T/C)GA(G/A)GGCTGCAAGGG(T/C)TTCTT-3′, and DBD210-238 (3′ primer), 5′-TCCTT(G/C)NGCATGCCCACTTCGA(G/A/T)GCACTT-3′. PCR amplifications were carried out by 30 cycles of 1 min of denaturation at 94°C; 1 min of primer annealing at 37°C (first 15 cycles) or 45°C (remaining cycles), and 2 min of

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Abbreviations: SON, supraoptic nucleus; RACE, rapid amplification of cDNA ends; DBD, DNA-binding domain; RXR, retinoid X receptor.

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¶The sequences reported in this paper have been deposited in GenBank [accession nos. L27513 (rat TR4) and L27586 (human TR4)].

extension at 75°C. At the final cycle, PCR mixtures were incubated at 75°C for 10 min to obtain full extension of all PCR products. A 147-bp PCR-amplified DNA fragment was further used as template to isolate full-length human and rat TR4 orphan receptor cDNAs (Fig. 1).

***In Situ* Hybridization.** *In situ* hybridization was performed with ^{35}S-labeled cRNA probes according to a well-established protocol for the brain (19) with minor modifications as outlined below. Cryostat sections (16 μm) from frozen rat brain were mounted on poly(lysine)-coated slides and stored at −80°C. Slides were placed at room temperature for 20 min and fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min. The slides were washed twice with PBS for 5 min and transferred to 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. This was followed by washing in PBS for 5 min and in 0.83% NaCl for 5 min. The slides were dehydrated slowly by immersing them in 30%, 50%, 70%, 85%, 96%, and 100% ethanol. The dehydrated slides were delipidated in chloroform for 1 min and washed in 100% ethanol again. The slides were air dried and used for hybridization on the same day.

An ^{35}S-labeled rat TR4 orphan receptor cRNA probe was synthesized by *in vitro* transcription. As controls for nonspecific binding, a 50-fold molar excess of unlabeled transcript was used to compete with the specific signal, and comparisons were made in parallel experiments with ^{35}S-labeled cRNA probes of similar G+C content for COUP-TF I and ARP-1. Hybridization was performed in 50% formamide/2× standard saline citrate (SSC)/10% dextran sulfate/1× Denhardt's solution/5 mM EDTA/10 mM phosphate buffer, pH 8.0/100 mM dithiothreitol containing yeast tRNA (0.5 mg/ml) and ^{35}S-labeled cRNA probe (10^6 cpm per section). The solution was heated at 80°C for 2 min and cooled on ice prior to being applied on slides (75 μl per slide). The slides were covered with coverslips, placed in a plastic slide box and incubated overnight at 50°C. Slides were then washed in 200 ml of 5× SSC/10 mM dithiothreitol at 65°C for 30 min for coverslips to fall off. High-stringency washing was performed in 50% formamide/2× SSC/10 mM dithiothreitol at 65°C for 30 min. This was followed by three 10-min washes in NTE buffer (0.5 M NaCl/10 mM Tris·HCl/5 mM EDTA, pH 8.0) at 37°C. The slides were treated with RNase A (20 μg/ml) in NTE buffer at 37°C for 15 min. The high-stringency wash in 50% formamide/2× SSC/10 mM dithiothreitol at 65°C was repeated. The slides were washed at room temperature in 2× SSC and in 0.1× SSC for 15 min each and then dehydrated quickly by putting them through 30%, 60%, 80%, and 95% ethanol containing 0.3 M ammonium acetate, followed by 100% ethanol, twice. Slides were then air dried and exposed to Kodak X-OMAT AR autoradiography film for 3 days. For microscopic analysis, the slides were dipped in Kodak NBT2 autoradiography liquid emulsion and exposed for 6 weeks or longer and counterstained with hematoxylin.

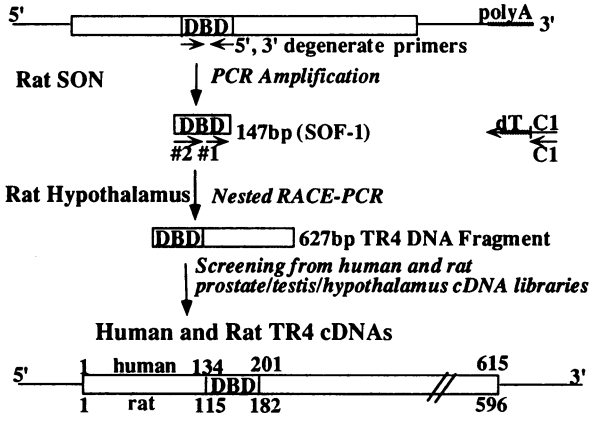

FIG. 1. Strategy of cloning full-length human and rat TR4 orphan receptor cDNAs. Two degenerate oligonucleotide primers 5' and 3' were used to amplify the DBD of TR4 orphan receptor from rat SON. The amplified 147-bp fragment (SOF-1) was used to design primers (#2, 5'-GGAGTGTGAGGAAGAATCTG-3'; #1, 5'-ACCTATAGC-TGTCGTAGCAG-3') for the nested rapid amplification of cDNA ends (RACE)–PCR (21) with primer C1 plus oligo(dT) (5'-AAGGATCCGTCGACATCGAT-TTTTTTTTTTTTTTT-3'). Three PCR products were obtained (TR4, TR4-β, and TR4-γ). The amplified 627-bp fragment of TR4 orphan receptor cDNA was then used as a probe to clone the full-length human and rat TR4 orphan receptor cDNAs (encoding 615 and 596 amino acids, respectively, as shown) from human prostate and testis as well as rat hypothalamus and prostate λgt11 libraries.

RESULTS AND DISCUSSION

PCR Amplification and Isolation of Full-Length cDNA for Human and Rat TR4 Orphan Receptors. Reverse transcription–PCR on RNA from microdissected SON tissue using degenerate primers directed to the DBD of steroid hormone receptors resulted in a single band of 145–180 bp. Since the two zinc fingers for most steroid hormone receptors are encoded by separate exons (20), this indicated that the PCR products were derived from spliced transcripts. Sequence analysis of PCR-amplified material cloned in a plasmid vector revealed an unknown sequence of 147 bp homologous to the DBD of the steroid hormone receptor superfamily that was encountered in 1 of every 5 clones. This product was tentatively indicated as supraoptic factor 1 (SOF-1, Fig. 1). Comparison of the predicted amino acid sequence of SOF-1 with other members of this superfamily revealed the highest degree of similarity (82%) with rat and human TR2 orphan receptor, an androgen-repressed orphan receptor isolated from prostate and testis (1, 6). To obtain further 3' sequence, nested RACE–PCR (21) with two primers from SOF-1 sequence was applied. Three PCR DNA fragments were obtained from rat hypothalamus, prostate, and testis. The 627-bp fragment revealed further homology to the TR2 orphan receptors. SOF-1 was therefore renamed as TR4 orphan receptor. This fragment was then used as a probe to clone full-length cDNAs by screening of human and rat prostate, testis, and hypothalamus λgt11 libraries.

The two additional PCR DNA fragments obtained by nested RACE–PCR (TR4-β of 969 bp and TR4-γ of 872 bp) overlapped partly with the 5' end of TR4 orphan receptor cDNA. The point of divergence corresponds precisely with a spliced junction in the human TR2 orphan receptor gene (unpublished data). TR4-β and TR4-γ harbor various stop codons and may well represent partially spliced transcripts of the TR4 orphan receptor gene.

Restriction enzyme mapping showed that isolated clones had overlapping sequences to form a 2.6-kb human TR4 orphan receptor cDNA and a 3.6-kb rat TR4 orphan receptor cDNA. The open reading frame between the first ATG and terminator TGA encodes 615 and 596 amino acids for human and rat TR4 orphan receptors with calculated molecular mass of 67.3 and 65.4 kDa, respectively (Fig. 2). Upstream of the first ATG codon is an in-frame terminator (TGA) indicating that this ATG may be a natural initiator in the rat TR4 orphan receptor. In the 3' untranslated region, a eukaryotic polyadenylylation signal (ATTAAA) was present at nucleotides 2222–2227 in human TR4 orphan receptor cDNA and at 2246–2251 bp in rat TR4 orphan receptor.

Human and rat TR4 orphan receptors have an identical amino acid sequence in the DBD with 19 nucleotide differences (Fig. 2). The homology in the C-terminal domains of human TR4 and rat TR4 is 98%. This region corresponds to the putative ligand-binding domain in other steroid receptors. Human TR4 and rat TR4 differ in the N-terminal region in 4

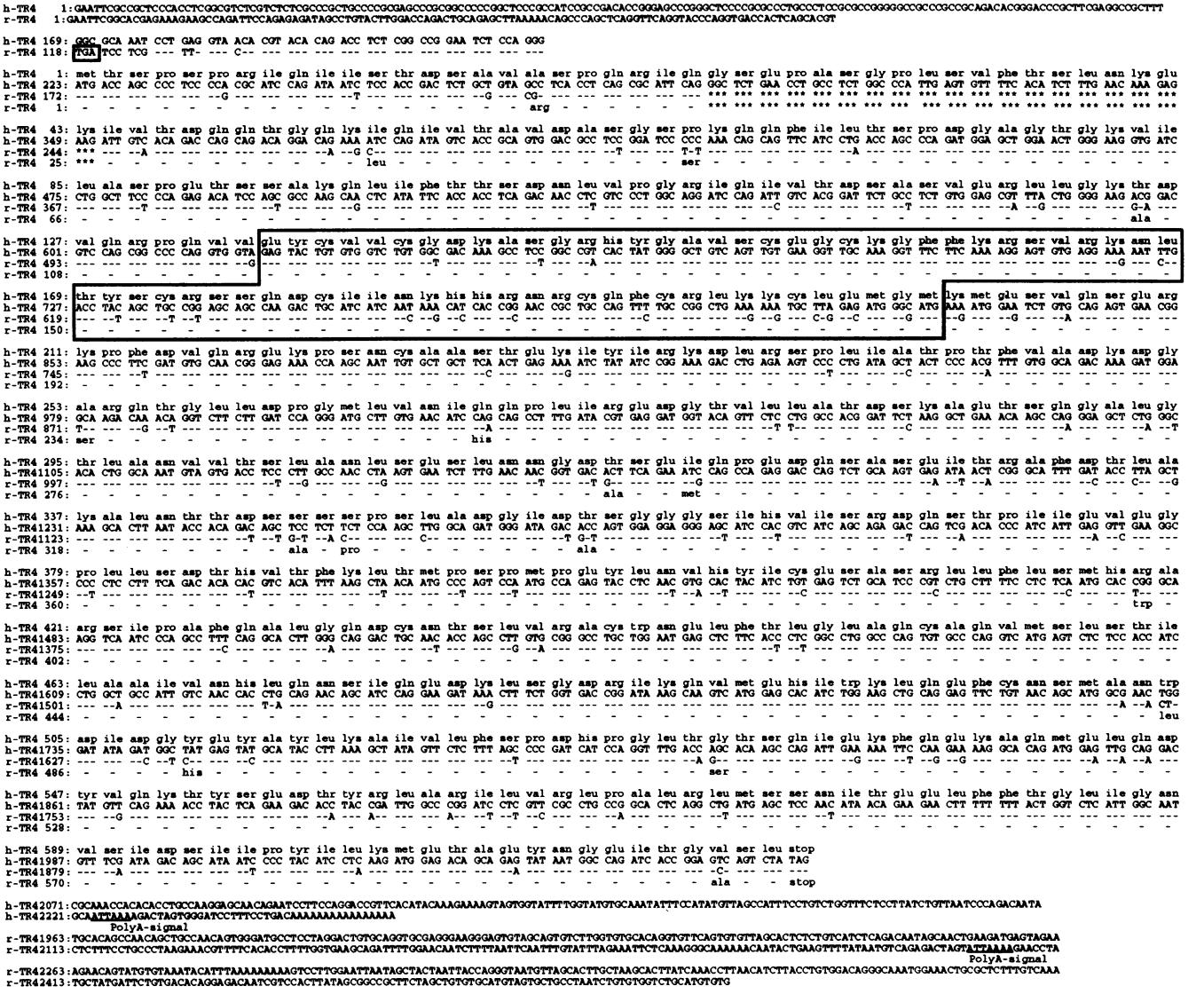

FIG. 2. cDNA sequences and deduced amino acid sequences of human (h) and rat (r) TR4 orphan receptors. The nucleotide sequences and the deduced amino acid sequences are numbered on the left. The rat TR4 orphan receptor residues that are identical with hTR4 orphan receptor residues are shown by hyphens. Gaps have been introduced to obtain an optimal match. The missing residues are shown by dots. The amino acid sequences in the putative DBD are boxed. An in-frame TGA terminator ahead of the first ATG of rat TR4 orphan receptor is also boxed and a eukaryotic polyadenylylation signal (ATTAAA) is underlined.

out of 114 amino acids. The overall homology between human and rat TR4 orphan receptors is 98%.

The most striking feature of the TR4 orphan receptor is the high homology with the TR2 orphan receptor (Fig. 3). The homology between these two orphan receptors in N-terminal, DBD, and ligand-binding domains is 51%, 82%, and 65%, respectively. The overall homology of 65% between TR4 and TR2 orphan receptors, which is higher than for any other member of the steroid receptor superfamily, indicates that TR4 and TR2 orphan receptors form a separate subclass of the superfamily. Examination of structural relationships of the DBD of the TR4 orphan receptor with that of other family members shows the segregation of TR2 and TR4 orphan receptors in a subclass that has the RXRs as closest relatives followed by the COUP-TF/ARP-1 subclass (Fig. 4). RXRs and COUP-TFs share their preference for binding to response elements composed of a direct repeat of the motif AGGTCA with 1-nucleotide spacing (DR1 element) (10, 11). It will be of interest to find the binding specificity of the TR4 orphan receptor to response elements in the target genes.

Size of TR4 Orphan Receptor mRNA and Its Tissue Distribution. The size of the TR4 orphan receptor mRNA was determined by Northern blot analysis (Fig. 5). One 8.4-kb band was detected in most of the examined tissues except for spleen (4.5 kb). The smaller size of the TR4 orphan receptor mRNA detected in spleen may suggest that two types of TR4 orphan receptor mRNA exist. The tissue distribution in rat showed that TR4 orphan receptor mRNA is most abundant in the rat central nervous system. Using hypothalamus as 100%, our Northern blot analysis showed the following relative amounts in other tissues: adrenal gland, 35%; spleen, 34%; testis, 33%; thyroid gland, 27%; prostate, 17%; and pituitary gland, 8%. No signal was detected on Northern blot for epididymis, seminal vesicle, submaxillary gland, heart, kidney, lung, and liver; PCR amplification, however, was able to detect a clear band from these tissues (data not shown). In a separate experiment, we were able to amplify a very strong TR4 orphan receptor signal from hippocampus.

In situ hybridization in rat brain showed labeling throughout the hypothalamic area (Fig. 6*A*) as well as the thalamic and cortical areas (Fig. 6 *A* and *B*), which indicated that TR4 orphan receptor is widely expressed in the brain. However, the most intense labeling for TR4 orphan receptor transcript was seen in distinct cell layers of the hippocampus and

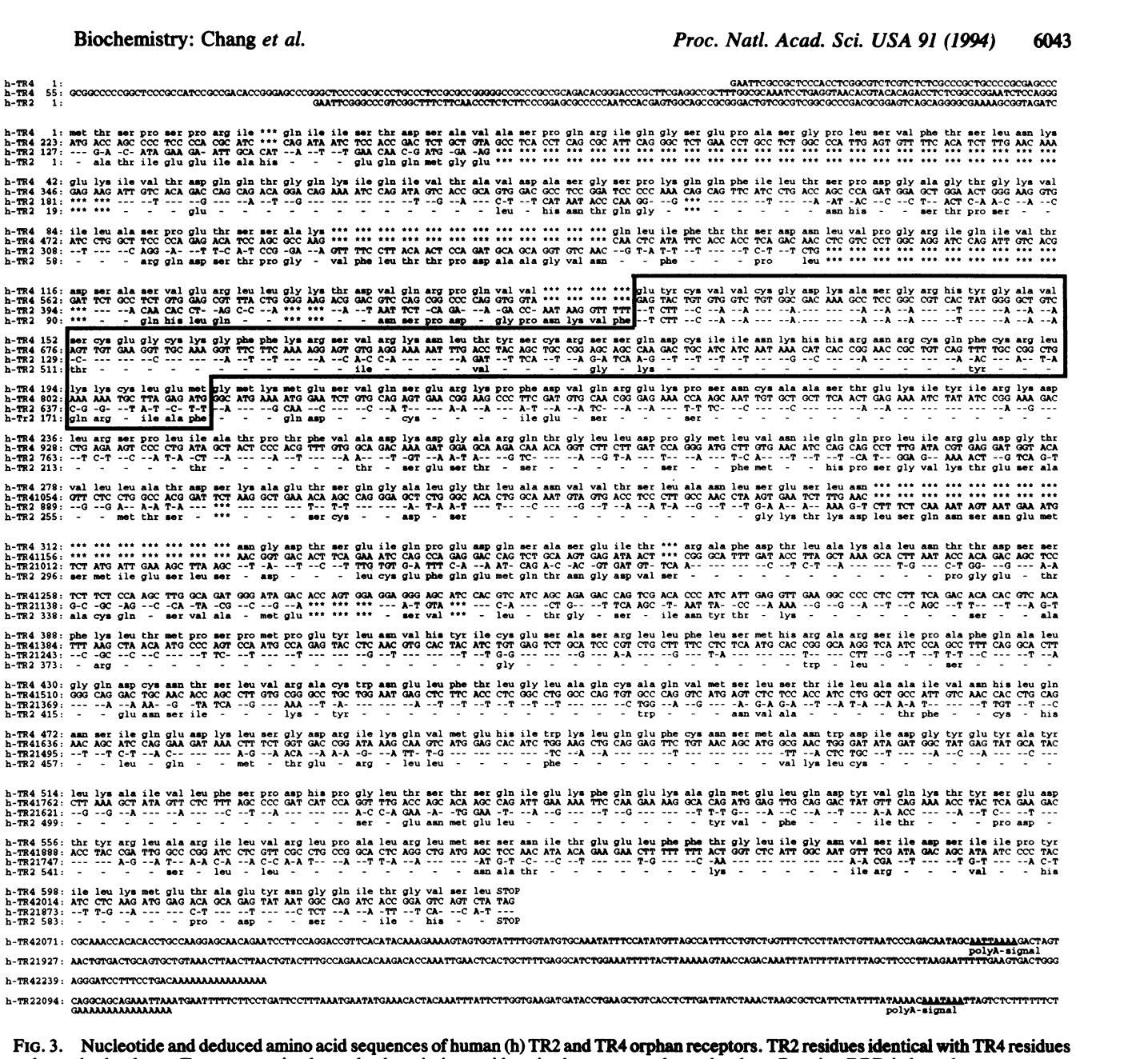

FIG. 3. Nucleotide and deduced amino acid sequences of human (h) TR2 and TR4 orphan receptors. TR2 residues identical with TR4 residues are shown by hyphens. To get a maximal match, the missing residues in the gaps are shown by dots. Putative DBD is boxed.

cerebellum (Fig. 6 *B* and *C*). Additional labeling of the habenula was seen (Fig. 6*B*). Two sets of control experiments were used to show the specificity of the *in situ* hybridization signal. First, in the presence of a 50-fold molar excess of antisense TR4 cRNA, no hybridization signal was obtained. Second, hybridization with ^{35}S-labeled cRNA probes for COUP-TF I and ARP-1, which were of similar G+C content and were hybridized under identical conditions, resulted in completely distinct hybridization patterns. Microscopic darkfield and brightfield examination of counterstained sections revealed that TR4 message was located in the granule cells in the hippocampus and cerebellum (Fig. 6 *D–G*).

The results indicate that TR4 orphan receptor transcripts do not exist only in the hypothalamus, the brain region from which it was initially amplified. It appears that TR4 is widely expressed at moderate levels throughout the brain and at high levels in granule cells of the hippocampus and cerebellum. These granule cells are small, densely packed neurons that have no direct functional properties in common. However, they share developmental aspects. Granule cells arise relatively late during brain development and undergo postnatal neurogenesis (22). It is not known whether they have a common embryonal origin. The high abundance of TR4 orphan receptor mRNA may be a consequence of the proliferation state of the granule-cell types in hippocampus and cerebellum or be related to a common regulatory function of TR4 orphan receptors in these cells. In either case, the abundant expression of the putative transcription factor, TR4 orphan receptor, suggests that these different neurons share certain cellular activities mediated by this orphan receptor. It is of interest that another gene (c-*src*) particularly associated with neuronal plasticity has a similar expression pattern. These properties may make the TR4 orphan receptor a suitable candidate to delineate functions for orphan receptors in the nervous system. As such, it may serve as a prototype for other orphan receptors, such as TR3/Nur77/NGFI-B and Nurr1, which are also present in the brain (9, 16) and may play a very important role in the nervous system.

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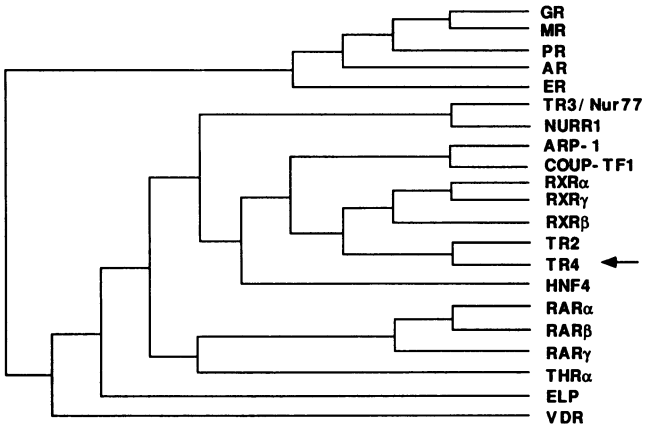

FIG. 4. Tree showing the structural relationship in the DBD of TR4 orphan receptor with other members of the steroid receptor superfamily. Amino acid sequences were compiled in the multiple sequence alignment program PILEUP (Genetics Computer Group sequence-analysis software). Human sequences were used except for NURR1 (rat), RXRγ (mouse), HNF4 (rat), and ELP (mouse). GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; ER, estrogen receptor; THR, thyroid hormone receptor; RAR, retinoic acid receptor; VDR, vitamin D receptor.

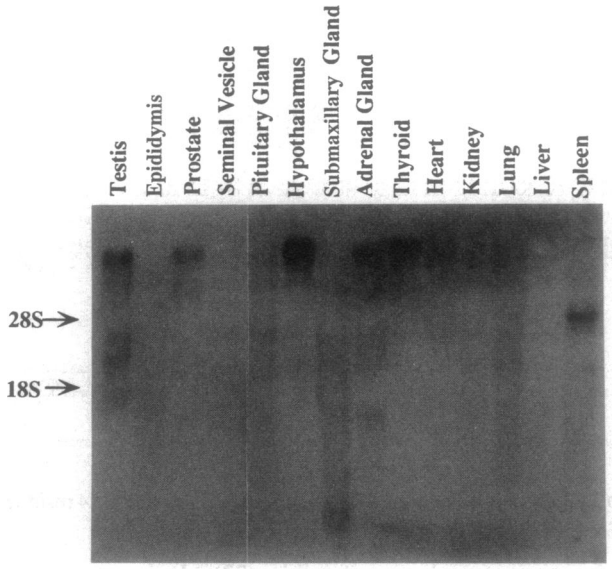

FIG. 5. Northern blot analysis of TR4 orphan receptor mRNAs from rat various tissues. Samples (20 μg) of total RNA were run in a 1.0% agarose gel with 2.2 M formaldehyde, transferred to a nylon filter, and probed with a ^{32}P-labeled rat TR4 orphan receptor cDNA. The blot was then washed, dried, and exposed for 16 hr with intensifying screens (20). Positions of 18S and 28S rRNA are indicated as size markers.

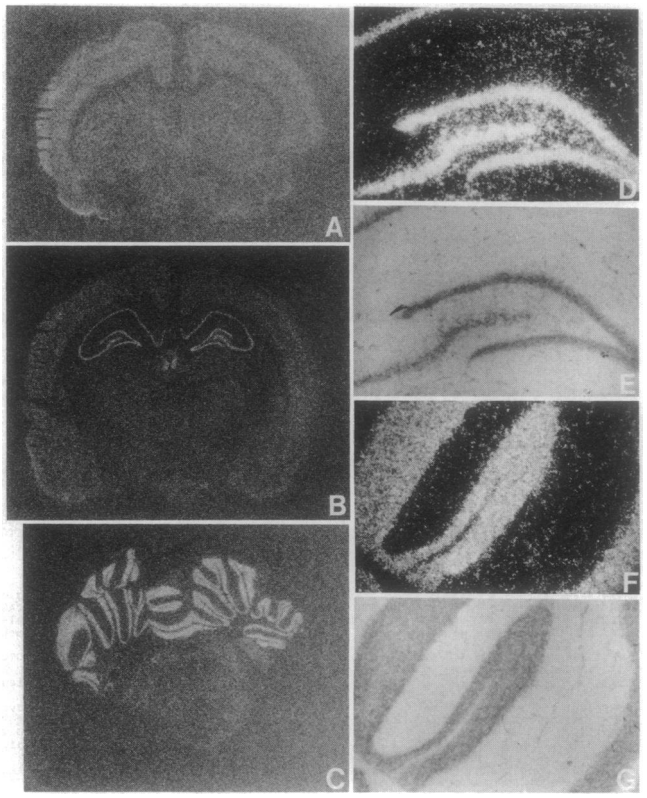

FIG. 6. Localization of TR4 orphan receptor transcripts in the rat brain by *in situ* hybridization. (*A–C*) Autoradiograms of transverse sections of the rat brain at the level of the hypothalamus (*A*), hippocampus (*B*), and cerebellum (*C*). Wide expression is seen in hypothalamic, thalamic, and cortical regions. Very intense hybridization signals are present in cell layers of the hippocampus and habenula (*B*) and in the cerebellum (*C*). (*D–F*) Darkfield (*D* and *F*) and brightfield (*E* and *G*) micrographs of the hippocampus (*D* and *E*) and the cerebellum (*F* and *G*). Localization of TR4 orphan receptors in pyramidal cells of the CA1, CA2, CA3, and CA4 layers and in granule cells is shown. [×3.5 (*A–C*); ×21 (*D–G*).]

1. Chang, C. & Kokontis, J. (1988) *Biochem. Biophys. Res. Commun.* **155**, 971–977.
2. O'Malley, B. W. (1990) *Mol. Endocrinol.* **4**, 363–369.
3. Power, R. F., Mani, S. K., Codina, J., Conneely, O. M. & O'Malley, B. W. (1991) *Science* **254**, 1636–1639.
4. Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. & Evans, R. M. (1992) *Nature (London)* **355**, 446–449.
5. Dreyer, C., Keller, H., Girel, F., Helftenbein, G. & Wahli, W. (1992) *Cell* **68**, 879–887.
6. Chang, C., Kokontis, J., Acakpo-Satchivi, L., Liao, S., Takeda, H. & Chang, Y. (1989) *Biochem. Biophys. Res. Commun.* **165**, 735–741.
7. Lydon, J. P., Power, R. F. & Conneely, O. M. (1992) *Gene Expr.* **2**, 273–283.
8. Chang, C., Kokontis, J., Liao, S. S. & Chang, Y. (1989) *J. Steroid Biochem.* **34**, 391–395.
9. Kokontis, J., Liao, S. & Chang, C. (1991) *Receptor* **1**, 261–270.
10. Cooney, A. J., Tsai, S. Y., O'Malley, B. W. & Tsai, M. J. (1992) *Mol. Cell. Biol.* **12**, 4153–4163.
11. Kliewer, S. A., Umesono, K., Hoyman, R. A., Mangelsdorf, D. J., Dyck, J. A. & Evans, R. M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1448–1452.
12. Mietus-Snyder, M., Sladek, F. M., Ginsburg, G. S., Kuo, C. F., Ladias, J. A. A., Parnell, J. E. & Karthanasis, S. K. (1992) *Mol. Cell. Biol.* **12**, 1708–1710.
13. Zhang, X. K., Lehmann, J., Hoffmann, B., Dawson, M. L., Cameron, J., Graupner, G., Hermann, T., Tran, P. & Pfahl, M. (1992) *Nature (London)* **358**, 587–590.
14. Miyata, K. S., Zhang, B., Marcus, S. L., Capone, J. P. & Ruchubinski, R. A. (1992) *J. Biol. Chem.* **268**, 19169–19172.
15. Adan, R. A. H., Cox, J. J., Beischlag, T. V. & Burbach, J. P. H. (1993) *Mol. Endocrinol.* **7**, 47–57.
16. Law, S. W., Conneely, O. M., DeMayo, F. J. & O'Malley, B. W. (1992) *Mol. Endocrinol.* **6**, 2129–2135.
17. Adan, R. A., Cox, J. J., van Kats, J. P. & Burbach, J. P. (1992) *J. Biol. Chem.* **267**, 3771–3777.
18. Palkovits, M. (1973) *Brain Res.* **14**, 449–450.
19. Bradley, D. J., Towle, H. C. & Young, W. S., III (1992) *J. Neurosci.* **12**, 2288–2302.
20. Chang, C., Saltzman, A., Lee, H., Uemura, H., Su, C., Chodak, G., Nakamoto, T., Beau, M. M. L., Espinosa, R., Davis, E., Lemons, R. S., Sivak, L. & Shih, C. (1993) *Endocrinol. J.* **1**, 541–549.
21. Graham, A., Hopkins, B., Powell, S. J., Danks, P. & Briggs, I. (1991) *Biochem. Biophys. Res. Commun.* **177**, 8–16.
22. Altman, J. & Das, G. D. (1965) *Nature (London)* **207**, 953–956.
23. Ross, C. A., Wright, G. E., Resh, M. D., Pearson, R. C. A. & Snyder, S. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9831–9835.